Method of Bottom-Up Directed Assembly of Cell-Laden Microgels

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Abstract—The paper describes a protocol to fabricate cell-laden microgel assemblies with pre-defined micro-architecture and complexity by a bottom-up approach, which can be used for tissue engineering applications. The assembly process was driven by hydrophobic effect in the water/oil interface. By agitating hydrophilic microgels in hydrophobic medium, the shape-controlled microgel units assemble in an organized manner to locally minimize the interaction free energy (the surface area exposed to the oil). The assembly process was shown to be controlled by several parameters, such as external energy input, surface tension, and microgel dimensions. This assembly approach was used to build multi-component cell-laden constructs by assembling microgel building blocks and performing a secondary cross-linking reaction. This bottom-up approach for the directed assembly of cell-laden microgels offers a scalable method to fabricate 3D tissue constructs with biomimetic structure.

Keywords—Bottom-up, Tissue engineering, Assembly, Hydrogel.

ABBREVIATIONS

DPBS Dulbecco’s phosphate buffered saline
MW Molecular weight
PEG Polyethylene glycol
UV Ultra violet

INTRODUCTION

This paper describes in detail the procedure for a bottom-up approach to assemble cell-laden microscale hydrogels (microgels) for fabrication of 3D tissue constructs. Bottom-up approaches build larger tissue constructs by the assembly of smaller cell-laden building blocks (i.e. microgels), which mimics the living tissue architecture from repeating functional units (i.e. islet, nephron or sinusoid). Thus far, bottom-up assembly of cell-laden microgels has been gaining increasing attention in the tissue engineering research, with numerous approaches developed including random assembly,6 manual manipulation,8 multi-layer photo-patterning,5,7 and microfluidic-directed assembly.4 Random assembly of microgel modules has the advantage of being rapid and simple, but lacks control over the final structure of the microgel assembly; manual manipulations are relatively slow processes and not scalable, multi-layer photo-patterning and microfluidic-directed assembly are able to create highly sophisticated microgel assembly architectures, but requires longer operational time and sophisticated equipments.

The bottom-up assembly process presented here aims to direct the assembly of cell-laden microgels in a simple and highly scalable manner. The assembly process is driven by the ‘hydrophobic effect’—the thermodynamic tendency of multiphase liquid–liquid systems to minimize the surface free energy between the phases (Fig. 1). Cell-laden microgels with defined sizes and shapes that were fabricated by photolithography were transferred into a hydrophobic mineral oil phase, and assembled with tunable micro-architecture upon application of a controlled agitation force. The cell-laden microgel assemblies could be further stabilized and harvested from the mineral oil for culturing in aqueous medium after a secondary cross-linking step. By assembling rectangular-shaped microgels, it was possible to control the overall dimensions and architecture of the assembly. To demonstrate the utility of this approach for generating more complex and ‘directed’ structures, a ‘lock-and-key’ design for the microgel shapes was used to control the relative position of two different types of microgels in the final assembly.

This approach requires the use of a hydrophobic phase such as mineral oil, which requires that living
cells be encapsulated in microgels to prevent direct exposure to the hydrophobic oil phase during the assembly procedure. In addition, it is still challenging to control the assembly three-dimensionally and achieve assemblies with uniform shapes (i.e. linear, branched and random assemblies were formed from assembling of square-shaped microgels).

The bottom-up approach assembly of microgels with defined 3D structures is a promising approach for engineering tissue constructs at large scale, which mimic the complexity of living tissues and opens a paradigm for directing the assembly of other mesoscale materials. By fabricating microgel building blocks with more complex geometries and properties and adoption of the secondary crosslinking, this approach can be potentially used to build biomimetic higher-order tissue construct that may be difficult and time-consuming to fabricate by using traditional tissue engineering methods. This protocol paper is expected to facilitate the application and further improvement of this approach.

FIGURE 1. Schematic representation of the microgel assembling. The steps of assembly process of microgel units are as follows: Synthesizing the microgel units by photolithography, transferring them into a petri dish filled with mineral oil, applying mechanical agitation through manual movement of the pipette-tip back and forth, exposing the microgel assemblies to UV-light for secondary crosslinking.

- Poly(ethylene glycol)-dimethacrylate polymer 1000 (Polysciences, Inc.; cat. no. 15178)
- Dulbecco’s Phosphate Buffered Saline (DPBS) (Gibco, cat. no. 14190)
- 1% photoinitiator (2-hydroxy-1-[4-(hydroxy-ethoxy)phenyl]-2-methyl-1-propanone) (Ciba Chemicals, Irgacure 2959)
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Preparation of Live/Dead Dyes
Add 2 μL of Calcein AM and 0.5 μL of Ethidium homodimer to 1 mL of DPBS.

PROCEDURE

Prepolymer Solution Preparation ● Timing 15 min
1. Mix 20% (w/w) of polyethylene glycol (PEG) with 80% (w/w) Dulbecco's Phosphate Buffered Saline (DPBS). Vortex until the PEG fully dissolves.
2. Add 1% of photoinitiator (2-hydroxy-1-(4-(hydroxyethoxy) phenyl)-2-methyl-1-propanone) to this solution. Vortex until the photoinitiator fully dissolves.

Note Dissolving the PEG first is essential, as the photoinitiator is difficult to dissolve otherwise.

Microgel Fabrication ● Timing 3 min/Slide
1. Design photomasks to control the desired shape of the microgels. Masks can be designed using programs such as Macromedia Freehand or AutoCAD and printed using a high resolution printer (with minimum 20,000-dpi resolution).
2. Place a drop of prepolymer solution (with the volume of 30 μL) in the middle of a base glass slide, then place spacer slides on the opposite of the base slide to control the height of the gels (the thickness of one spacer slide is 150 μm). To adjust the height of the gels, different numbers of spacer slides can be combined. The size of the drop should be adjusted to ensure prepolymer does not spill over the edge when a cover glass slide is placed above the solution.
3. Shine UV light on the device to induce gel formation. The exposure time must be adjusted depending on the size of the features in the gel. For a 400 × 400 × 150 μm microgel, a UV exposure of 12.4 mW cm⁻² at 360–480 nm for 30 s was sufficient for formation.
4. Separate the top and bottom slides to allow for microgel collection.

Note If the gels will not be used right away, they should be kept under DPBS to avoid adherence to the glass slides.

Microgel Assembly ● Timing 10 min
1. Fill a 60 × 60 × 15 mm Petri dish with 6 mL of mineral oil.
2. Collect the microgels into a small cluster. Dry all excess liquid and then drop a minimal amount of prepolymer solution on the microgels (~5 μL).

EQUIPMENT
- Photomasks with different patterns (i.e., rectangular or lock-and-key) designed using AutoCAD and printed on transparencies with 20,000-dpi resolution (CAD/Art Services)
- Micro cover glasses (150 μm thick, 18 × 18 mm)
- UV light (The OmniCure S2000 UV/Visible Spot Curing System)
- 60 × 15-mm dish (Fisher Scientific, cat. no. 430589)
- 10 mL pipette tips
- 15-mL tubes (BD Biosciences, cat. no. 352096)
- Vortexer
- Centrifuge
- Incubator (5% CO₂ at 37 °C)
- Microscope (with 4× and 10× objective lenses)

REAGENT SETUP

Preparation of Prepolymer Solution
Dissolve 20% (wt/wt) poly(ethylene glycol)-methacrylate polymer in Dulbecco’s Phosphate Buffered Saline. Add 1% (wt/wt) photoinitiator before UV polymerization.

Preparation of Cells for Encapsulation
Add 1× trypsin to cells in the flask and resuspend the cells in the prepolymer solution at a concentration of 1 × 10⁷ cells/mL.
3. Transfer the microgels to the mineral oil filled
dish.
4. Sketch straight lines with a thin pipette tip (1–200 µL) through the cluster of microgels. Continue
this for 60 s with an agitation rate of 36 cm s⁻¹
(Corresponding to Reynolds number of 3).

**Note** If lines are sketched too slowly, ordered assembly will not occur.

5. Place the entire dish under UV light again to
stabilize the structures. A shorter time can be
used for this stabilization (using the previously
mentioned conditions, 4 s is sufficient for stabil-
ization).

**Note** If an excess of hydrophilic liquid remains in the
gel cluster, assembly will not occur as effectively. If
microgels do not assemble properly, try drying the
cluster more.

**Cell-Laden Microgel Assembly ● Timing 60 min**

1. Isolate a sufficient number of cells for all experi-
ments. Typically, a cell density of 1 × 10⁷ cells/mL
of prepolymer solution was used.
2. Obtain a cell pellet by centrifuging at 800 rpm and
remove excess cell medium. Re-suspend the pellet in
prepolymer solution.
3. Repeat the ‘Microgel fabrication’ and ‘Microgel
assembly’ procedures. Ensure the solution and cells
are well mixed by pipetting the mixture up and
down several times prior to the fabrication of each
slide.

**Note** If solution is not well mixed, cells will sink to the
bottom of the solution and hence not be well distrib-
uted in the gels.

**Note** Avoid making bubbles in the solution by
keeping the pipette tip submerged when pipetting up
and down.

4. After secondary UV exposure, remove excess
mineral oil from the dish with a pipette. Wash the
dish with cell culture medium 3 times to remove
more oil and then submerge the structures in
medium.

**Note** Take caution and avoid microgels when aspirat-
ing the mineral oil and culture medium.

**Timing**

Prepolymer solution preparation (15 min)
Microgel fabrication (3 min/slide)
Microgel assembly (10 min)
Cell-laden microgel assembly (60 min)

**Troubleshooting.**

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible reason</th>
<th>Suggested solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microgels do not form</td>
<td>Mask size is too small</td>
<td>Increase the feature sizes</td>
</tr>
<tr>
<td>Microgels were broken</td>
<td>Insufficient UV exposure</td>
<td>Increase UV time</td>
</tr>
<tr>
<td></td>
<td>Agitation too harsh</td>
<td>Decrease Reynolds number by decreasing agitation rate</td>
</tr>
<tr>
<td></td>
<td>Pipette tip too sharp</td>
<td>Use a larger pipette tip</td>
</tr>
<tr>
<td>Failure of secondary crosslinking</td>
<td>Prepolymer was washed away prior to second crosslinking</td>
<td>Add a small amount of prepolymer to the microgel cluster</td>
</tr>
<tr>
<td></td>
<td>Not enough secondary UV exposure</td>
<td>Increase secondary UV exposure time</td>
</tr>
<tr>
<td>Cells in the gel are dead</td>
<td>Excessive UV exposure</td>
<td>Decrease first and/or second UV exposure (though the gels and aggregates must still be formed)</td>
</tr>
</tbody>
</table>

**ANTICIPATED RESULTS**

By using the current protocol, microgels with
different dimensions from 200 to 1000 µm with
200 µm increments (for rectangular microgels) and
different shapes (lock & key) could be assembled. In
case of rectangular microgel assemblies made with
microgels of different sizes, it was observed that the
average length of the linear microgel assemblies has
direct relation with the aspect ratios of the
microgels. To be more specific, the obtained aspect
ratio of microgel assemblies is in proximity of 1
(Fig. 2a).

In order to show the feasibility of this technique for
generation of more sophisticated and ‘directed’ struc-
tures, the microgel shapes was designed in a ‘lock-and-
key’ pattern. The ‘lock-and-key’ design enables us to
locate two different types of microgels in a desired
location in the final assembly (Fig. 2b).

To stabilize the interaction between assembled
microgel structures (Fig. 2c), a secondary crosslink-
ing was applied. This step was performed after the
formation of microgel assemblies. It is also notice-
able that the residual prepolymer solution sur-
rounding the individual microgels prior to agitation
was necessary for the success of the secondary
crosslinking. As a demonstration of the use of the
micro-scale hydrogel assembly process developed
here for biological applications, NIH-3T3 fibroblasts
were encapsulated within the individual microgels
and assembled into linear structures (Fig. 3a). A high
fraction of the cells remained viable immediately
We further demonstrated the applications of the lock-and-key directed assembly for generating cellular co-cultures. Two different cell types stained by red and green cell tracker respectively were encapsulated in cross-shaped or rod-shaped microgels (Fig. 3c) and assembled by this approach. Microscale tissue constructs composed of two cell types were fabricated, which can be readily used as co-culture system.

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